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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 13/00, C12N 15/18
A61K 37/36

A1

(11) International Publication Number: WO 90/08164

(43) International Publication Date: 26 July 1990 (26.07.90)

(21) International Application Number: PCT/US89/05447

(22) International Filing Date: 11 December 1989 (11.12.89)

(30) Priority data:
299,107 19 January 1989 (19.01.89) US

(60) Parent Application or Grant
(63) Related by Continuation
US 299,107 (CON)
Filed on 19 January 1989 (19.01.89)

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, ES (European patent), FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU, US.

Published
With international search report.

(54) Title: SOMATOTROPIN ANALOGS

(57) Abstract

The present invention provides analogs of mammalian somatotropins wherein the asparagine residue of position 99 of the native mammalian somatotropin is replaced with a compound selected from the group consisting of proline, aspartic acid, glutamic acid, serine, glycine, serine-serine or serine-aspartic acid.

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SOMATOTROPIN ANALOGS

FIELD OF INVENTION

This invention relates to analogs of animal somatotropins or growth hormones. More specifically, the invention relates to novel bovine growth hormone analogs, particularly with changes in amino acid residue 99.

BACKGROUND OF THE INVENTION

Bovine somatotropin (bSt) is a growth hormone that has been well studied (Paladini, A.C. et al., CRC Crit. Rev. Biochem. 15:25-56 (1983)). Somatotropins were originally discovered in pituitary gland extracts from various animals. In general, somatotropins are conserved molecules and similarities in amino acid sequences and structure are found between different species of animals.

Somatostatins, including bSt, are globular proteins comprising a single chain of about 200 amino acids with two intramolecular disulfide bonds. Specifically, bSt has a single 190-191 amino acid chain, a globular structure with two intramolecular disulfide bonds and a molecular weight of about 22,000 daltons.

Natural bSt extracted from pituitary glands is heterogeneous. At least six major forms of the protein have been described. The longest form has 191 amino acid residues and an ala-phe amino terminus. The second form has 190 amino acid residues and a phe amino terminus. The third form has 187 amino acid residues and a met amino terminus. The remaining three forms of bSt substitute valine for leucine at position 127. In addition to this heterogeneity, undefined heterogeneity of bovine somatotropin has also been described (Hart, I.C. et al., Biochem. J. 218:573-581 (1984); Wallace, M. and Dickson, H.B.F., Biochem. J. 100:593-600 (1965)).

Undefined electrophoretic heterogeneity is seen when the native extracts are fractionated by anion exchange chromatography. It has been shown that the defined forms have different relative potency in bioassays. Also, it has been shown that other undefined species of bSt when fractionated on ion exchange columns demonstrate varying degrees of bioactivity in rat growth models (Hart, et al. and Wallace and Dickson, supra).

It is not known whether undefined heterogeneity exhibiting biological variation is due to genetic variability, to in vivo post-translational modification, to differences in phosphorylation

(Liberti, J.P. et al., Biochem. and Biophys. Res. Comm. 128:713-720, 1985), or to artifacts of isolation.

Bovine somatotropin produced by recombinant microorganisms (rbSt), or extracted from pituitary gland tissue, is important commercially. It increases lactation in dairy cattle and increases size and meat production in beef cattle. It is estimated that upwards to 20 mg per animal per day will be needed to effect commercially acceptable improvements in production. Such a dosage will require efficient methods of administration. Improvements in the potency and stability of bSt such as described in this invention will be of benefit because of resulting reductions in the amount of drug administered to each animal per day.

Furthermore, one of the problems in preparing recombinantly-produced bSt is that liquid processing and storage of rbSt at acid or alkaline pH results in the conversion of the asparagine residue at position 99 to isoaspartic acid. The resulting rbSt is referred to as "early eluting rbSt" because it elutes earlier than native rbSt on reversed phase HPLC. Isoaspartate is formed when the asparagine side chain condenses with the peptide backbone resulting in the elimination of ammonia. Chain cleavage also occurs by a condensation reaction between the peptide backbone and the asparagine residue at position 99 of the rbSt molecule upon storage. The chain-cleaved product is covalently held together by the disulfide bond between cysteine residues 53 and 164 and has been called "early-early eluting rbSt" because of its eluting position relative to native and early eluting rbSt.

Because the instability that occurs due to the modification of asparagine 99 leads to a loss of native rbSt during its isolation, formulation and storage as reconstituted product, it would be advantageous to make an amino acid substitution at position 99 to produce an rbSt analog that is more stable while retaining or enhancing its biological activity.

INFORMATION DISCLOSURE

Analogs of bSt are known (see, for example, European patent applications 75,444 and 103,395 and Nucleic Acid Res. 10(20):6487 (1982)).

G. Winter and A.R. Fersht, TIBS, 2, p. 115 (1984) review the alteration of enzyme activity by changing amino acid composition at

key sequence locations.

P.Y. Chou and G.D. Fasman, Ann. Rev. Biochem., 47, pp. 251-76 (1978) refer to the use of amino acid sequences to predict the secondary and tertiary structure of proteins. P.Y. Chou and G.D. Fasman, J. Mol. Biol., 115, pp. 135-75 (1977) refer to β-turns in proteins. From analysis of 459 β-turn regions in 29 proteins of known sequence and X-ray structure, they found that the most frequently occurring amino acids in the third position of a β-turn are asparagine, aspartic acid, and glycine. The residues with the highest β-turn potential in all positions within the turn are proline, glycine, asparagine, aspartic acid, and serine.

SUMMARY OF THE INVENTION

This invention relates to the enhancement of bioactivity or stability in liquid storage, or both, of bSt and analogs thereof, by substituting different amino acids for the asparagine corresponding to the residue at position 99 of native bSt. Similar changes can be made in somatotropins from other animals, particularly mammals, including porcine, fish, ovine, horse, rat, monkey, and human.

More specifically, and preferred, are those species of bSt-like compounds wherein the asparagine located at amino acid residue 99 is replaced with a different amino acid residue including specifically glycine, serine, proline, aspartic acid, glutamic acid, serine-serine or serine-aspartic acid.

More specifically, the animal somatotropin is selected from the group consisting of bovine, porcine, fish, ovine, horse, rat, monkey, and human somatotropins.

Even more specifically, the animal somatotropin is bovine somatotropin.

Also provided is a method for enhancing the growth of an animal, particularly a mammal, and more particularly a bovine, which comprises administering to the animal an effective amount of a somatotropin of the instant invention, and, in particular, where the animal is a bovine and the somatotropin is bSt.

Also provided is a method for increasing milk production in a female ruminant comprising administering to the female ruminant an effective amount of an animal somatotropin of the instant invention, and, in particular, where the animal is a bovine and the somatotropin is bSt.

DETAILED DESCRIPTION

Due to the molecular heterogeneity of somatotropins, the position numbers of amino acid residues of the various somatotropins may differ. The term "native mammalian somatotropin" includes these naturally occurring species. Chart 1 illustrates the specific region of one species of bSt that corresponds to the position 99 residue modified by this invention. The numbering for other somatotropins may differ where other species or analogs are involved. Using the asparagine 99 of the bSt set forth in Chart 1, those of ordinary skill in the art can readily locate corresponding amino acids in alternative animal somatotropins, for example, mammalian somatotropins, for example, asparagine 99 of mammalian somatotropin, or their analogs, to achieve the desired liquid storage stability, enhanced bioactivity and uniform potency of the instant invention.

Both chemical and genetic modifications of this region are embraced by this invention.

The preferred genetic modifications rely upon single site specific mutation methods for insertion of various amino acid residues in replacement of the naturally occurring asparagine.

The phrase "animal somatotropin" refers to somatotropins originating from animals, e.g., mammals, and includes somatotropins derived from either natural sources, e.g., pituitary gland tissue or from microorganisms transformed by recombinant genetics to produce a naturally-occurring form of somatotropin. When a specific mammalian source is named such as a bovine somatotropin or a somatotropin of bovine origin, the somatotropin includes those derived from either natural sources or from transformed microorganisms.

The term "microorganism" is used herein to include both single cellular prokaryotic and eukaryotic organisms such as bacteria, yeast, actinomycetes and single cells from higher plants and animals grown in cell culture.

The term "native" refers to naturally-occurring forms of somatotropins which may have been derived from either natural sources, e.g., pituitary gland tissue or from microorganisms transformed by recombinant genetics to produce a somatotropin having the same amino acid sequence as the naturally-occurring form of somatotropin.

The mammalian somatotropins are very similar in amino acid

sequence and physical structure. Although the processes described in the Examples are directed toward bSt, the processes are equally applicable to any animal, e.g., mammalian somatotropin having the requisite asparagine residue available for replacement particularly wherein similar liquid processing and storage problems are encountered.

The high relative potency of the bSt analogs of the present invention is readily determined using hypophysectomized rats. Evans, H.M. and Long J.A., Anat. Rec. 21:61, 1921. Relative increases in total body weight are recorded using pituitary bSt, recombinant bSt (rbSt) and various bSt analogs of the invention.

Site-Directed Mutagenesis: Several techniques for site-directed mutagenesis have been developed for introducing specific changes in a DNA sequence and may be used to produce the compounds of the instant invention (Kramer, W., et al, Nucl. Acids Res., 12, pp. 9441-56 (1984); Mandecki, W., Proc. Natl. Acad. Sci. USA, 83, pp. 7177-81 (1986); Zoller, M.J. and Smith, M., Nucl. Acids Res., 10, pp. 6487-6500 (1982); Norrander, J., et. al., Gene, 26, pp. 101-106 (1983); Kunkel, T.A., Proc. Natl. Acad. Sci. USA, 82, pp. 488-92 (1985); Schold, A., et. al., DNA, 3, pp. 469-77 (1984)). We employed the primer directed mutagenesis technique of Schold et al for five of the seven analogs produced, except that only one primer is used for the initial hybridization reaction and 18 μl of T_4 gene 32 protein is added to the extension reaction.

Colony Filter Hybridization: The screening technique of filter hybridization is based upon the ability of a single-stranded segment of DNA to locate its complementary sequence and hybridize to form a double-stranded segment, Hanahan, D. and Meselson, M., Meth. Enzymol., 100, pp. 333-42 (1983). The thermal stability of this binding is dependent upon the number of matches and mismatches contained within the double stranded region. The more mismatches it contains, the weaker the base-pair binding and the lower the temperature necessary to disrupt the DNA binding. This temperature differential is exploited during colony filter hybridization, Bryan, R., et. al., Microbiology (1986). By constructing a mutant oligomer which maximizes the temperature differential between the native and mutant sequence, it is possible to hybridize at a lower temperature allowing binding of the probe to matched and nearly matched sequen-

ces. Upon washing at elevated temperatures, the mismatched probe-DNA duplex becomes unstable and disassociates while the perfectly matched duplex remains bound. The matched duplex will then produce the darkest signal on an autoradiogram thus forming a detection method for a colony containing the desired sequence. DNA from this colony can then be isolated and sequenced.

For filter preparation, nitrocellulose filters are overlayed onto plates and wetted. The filters and plates are marked for orientation and the filters are then carefully lifted off the plates. The master filter plates are incubated overnight at room temperature to allow re-growth of the colonies. The filters are denatured by laying them one by one onto Whatman paper soaked in 0.5 M NaOH, 1.5 M NaCl for 10 minutes and neutralized in two successive changes of Whatman paper soaked in 1 M Tris, pH 7.4, 1.5 M NaCl, for 10 minutes each and air dried on fresh Whatman paper for 30 minutes. They are then baked for 2 hours at 80°C in vacuum.

The kinase reaction to radiolabel the mutant oligonucleotide for use as a probe is as follows: 2 μg of oligo, 2 μl of 10X kinase buffer, 100 μCi γ32-P ATP, 2 μl T4 kinase and 4 μl water are mixed and incubated for 1 hour at 37°C. A 1 ml column is packed with DEAE-Sephacel in a 10 ml disposable column and equilibrated with 2-3 ml of high salt buffer (1.5M NaCl in TE) and then 2-3 ml of low salt buffer (0.2M NaCl in TE). The kinase reaction is diluted with 200 μl of low salt buffer and loaded directly into the column. The column is washed with 10 ml of low salt buffer until no further counts elute from the column. The probe is eluted in 4 ml of high salt buffer.

To hybridize, the filters are placed in a crystallization dish and batch pre-hybridized in 5X Denhardts (1% BSA, 1% Ficoll and 1% PVP), 5X SSC (0.75 M NaCl, 0.075 M sodium citrate) and 0.1% SDS for 1 hour at 40°C. The hybridization solution is changed and the probe is added. The dish is covered and the hybridization done overnight with gentle agitation. The filters are then rinsed with several changes of 5X SSC, 0.1% SDS. The filters sit in this solution while the water bath and wash solution (5X SSC, 0.1% SDS) is heated up to washing temperature (46°C). The filters are transferred one by one to a fresh crystallization dish and washed 3 x 20 minutes, changing dishes after each wash. They are then air dried on Whatman paper, wrapped in Saran wrap and exposed as necessary.

Vector DNA Preparation: DNA for sequencing is obtained according to the method of L. Agellon and T. Chen, Gene Anal. Techn., 3, pp. 86-89 (1986) except that only one combined phenol/chloroform extraction is performed and the DNA is not spin-dialyzed through a Sephadex G-50 column.

Sequencing: Double-stranded sequencing is performed according to the following protocol: 3 µl 2N NaOH, 2 mM EDTA is added to 12 µl of DNA (2 µg) and incubated for 15 minutes. 6 µl 3 M NaOAc, 1 µl primer and 100 µl 95% ethanol are added and the DNA precipitated on dry ice for 20-30 minutes. The pellet is collected, washed and vacuum dried. It is dissolved in 13 µl water and 4 µl RT buffer (0.3 M Tris-HCl, pH 8.3, 0.375 M NaCl, 37.5 mM $MgCl_2$, 2.5 mM DTT), 2 µl γ32P dCTP and 1 µl reverse transcriptase are added. 4 µl of this mix is pipetted into 4 eppendorf tubes, each containing 1 µl of G mix, A mix, T mix or C mix. The tubes are incubated for 10 minutes at 42°C. 1 µl of chase mix (0.25 mM dNTPs) is added and they are incubated for an additional 5 minutes. 10 µl stop solution (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromphenol blue) is added, the reactions are boiled 3 minutes and 3 µl of each is loaded onto a sequencing gel.

Induction Protocol and SDS-PAGE Analysis: See PCT/US 88/00328.

Example 1

A site-directed mutagenic technique for double-stranded primer extension is used to introduce altered codons for serine and proline at amino acid position 99 in the rbSt cDNA m4 gene (PCT patent application PCT/US 88/00328, filed 27 January 1988 and incorporated herein by reference). In this method, the target sequence is cloned into a suitable plasmid and plasmid DNA is prepared. The plasmid DNA is denatured by treatment with NaOH which causes "nicks" in the DNA molecule deoxyribose-phosphate backbone. This relaxes the DNA and permits an oligomer containing the desired sequence changes to hybridize to the plasmid sequence containing the position 99 residue of bSt. The 3′ end of the oligomer generates a primer for the DNA polymerase activity of the reverse transcriptase which extends the primer, synthesizes a new DNA strand containing the mutagenic oligomer and displaces the normal complementary strand. The extension reaction increases the probability of the incorporation of the oligomer-directed change. The DNA is transformed into competent

cells and the resultant colonies are screened by colony filter hybridization. Plasmid DNA is isolated and sequenced from positive candidates.

The oligomers used to construct the position 99 serine and proline changes in the rbSt m4 gene are produced by techniques previously described (PCT/US 88/00328). An oligonucleotide so produced and designated CST-88 (Chart 3) contains the change on the DNA sequence asparagine AAC to serine TCT and another designated CST-89 (Chart 3) contains the asparagine AAC to proline CCG change. They are both designed with a Tm of 53°C thus allowing for hybridization at 40°C and stringent washing at 46°C as set forth above.

In parallel experiments the serine and proline oligomers are hybridized to the pBR322 derived vector pTrp-BStm4. This vector contains the trp promoter and the m4 cDNA for rbSt (PCT/US 88/00328). After primer extension, the DNA is used to transform competent cells of MC1000 (available in the Experiments with Gene Fusion Strain Kit, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The transformed cells are plated to give 100-200 colonies on ten plates. The colonies are then transferred onto nitrocellulose. The cells are lysed and their DNA affixed to the filter. Radiolabeled oligomer probes are prepared from CST-88 and CST-89 by the oligomers with kinase and 32P-ATP. Details of the colony filter hybridization probing are set forth above.

Six candidates gave a very strong positive signal on the autoradiogram for the serine mutagenesis. Plasmid DNA was isolated for each candidate and sequenced. One of the candidates was found to contain the serine change. The mutated gene contained therein is designated m4-99ser. For the proline mutagenesis, six positive candidates were analyzed by DNA sequencing and two were found to contain the desired change. These mutated genes are designated m4-99pro.

The m4-99ser and m4-99pro genes are excised from the parental vector as an EcoRI-HindIII fragment and cloned into the EcoRI-HindIII restriction sites of the pURA-m4 vector (PCT/US 88/00328). Chart 2 shows the cloning of the m4-99ser gene into the pURA-m4 vector. The identical construction is carried out for the m4-99pro gene. Upon sequence confirmation of the clonings, the vectors are designated pURA-99Ser and pURA-99Pro. These vectors are transformed into

fermentation expression strain BST-1C (PCT/US 88/00328).

Transformed cells from each of the clonings are induced and samples analyzed by SDS-PAGE to assess the ability of the cells to express the modified rbSt genes under non-optimized conditions. Results of SDS-PAGE analysis showed that pURA-99Ser produced rbSt in three individual inductions at 13.8%, 14.6% and 26.4% of total cellular protein. Results of SDS-PAGE analysis showed pURA-99Pro in four separate inductions expressed rbSt at 28.2%, 29.6%, 34.0% and 41.9% of total cellular protein.

Because of poor mutagenic efficiency, the mutant oligomer must be constructed so that there is at least a 5°C temperature difference between its Tm and that of the native sequence. Without this difference, effective screening of the candidates cannot be accomplished.

Example 2

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (C-ST 90 and C-ST 91, Chart 3), bSt analogs having aspartic acid and glutamic acid at position 99 were also constructed.

Example 3

Analogs having two amino acids substituted in place of the position 99 asparagine (Ser-Ser and Ser-Asp) were constructed by following the site directed protocol of Kramer et al, Nucl. Acids Res., 12, p. 9441-56 (1984) as described in the "Site Directed Mutagenesis Kit", commercially available from Boehringer Mannheim Biochemicals, PO Box 50816, Indianapolis, IN 46250 (see also, Kramer, W. and H-J Fritz, Meth. Enz., 154, pp. 350-67 (1987)). The procedure requires cloning the DNA sequence which is to be modified into the M13mp9 vector. This is done by digesting vector pURA-m4 (PCT/US 88/00328) with the restriction enzymes EcoRI and BamHI and isolating a DNA fragment approximately 870 bp in size. This fragment contains the *E. coli* tryptophan (trp) promoter, the trpL ribosome binding site and the entire bSt gene sequence. This fragment is cloned into the EcoRI and BamHI restriction sites of the M13mp9 vector using known techniques (Maniatis et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Publication, Cold Spring Harbor, New York; J. Messing, Meth. Enzym., 101, pp. 20-98 (1983)). Single stranded DNA is isolated using the derived vector M13mp9-m4 (Messing, supra) and used

in the mutagenesis procedure as previously described. Cloning of these analogs into the pURA expression vector was done as described in Example 1, in which the double-stranded DNA of the M13 vector, supra, is substituted for the pBR322 vector.

Example 4

A position 99 analog having glycine substituted for asparagine was also constructed using the techniques described in Example 1. The mutagenesis oligonucleotide used is designated C-ST84 (Chart 3). It encodes the change to glycine.

Example 5

Activity of various position 99 analogs was demonstrated. The parameter measured was to estimate differences in 3.5% fat corrected milk yield (FCM) of lactating dairy cows injected intramuscularly with native rbSt, the glycine-99 (Gly-99) rbSt of Example 4, the serine-99 (Ser-99) rbSt of Example 1 and the aspartic acid-99 (Asp-99) rbSt of Example 2. Holstein cows (45) were ranked from high to low milk yield based on milk yield on days 3 and 2 prior to initiation of rbSt injections. The cows were assigned randomly in replicates, based upon milk yield, to 9 experimental groups: no injection (Control), 5 mg and 20 mg Gly-99 rbSt daily, 5 mg and 20 mg Ser-99 rbSt daily, 5 mg and 20 mg Asp-99 rbSt daily, and 5 mg and 20 mg native rbSt daily. Cows were injected imtramuscularly in the semitendinosus muscle once daily for 21 days. Twice daily milk weights were recorded for three days prior to initiation of injection, during the 21 days of injections, and for five days after the last injection. Concentration of rbSt was expected to be 10 mg/ml.

The concentration of residual post-injection rbSt solutions, measured by HPLC, averaged 11.5 for native rbSt, 12.1 for Asp-99 rbSt, 11.6 for Gly-99 rbSt, and 11.6 for Ser-99 rbSt. The area percent was greater than 99% normal rbSt for Gly-99 rbSt (by "normal rbSt" is meant the amino acid sequence encoded by a particular gene, i.e., no degradation products, etc.), for Ser-99 rbSt, and for Asp-99 rbSt, but averaged 90.5 normal rbSt for native rbSt. Native rbSt averaged 2% early-early eluting rbSt and 7.6% early eluting rbSt.

Statistical analyses of the FCM among experimental groups were based on average FCM for days 1 to 21 of injections using the three days prior to initiation of injections as the covariate. Average daily FCM (kg/day) was not statistically significantly different for

either form of rbSt (P<.12) or dose X form of rbSt interaction (P<.90). However, since the statistical significance was P<.12 for form of rbSt, an attempt was made to gain additional information on the relative potency among the forms of rbSt. Based on comparisons among the four forms of rbSt, there was a suggestion (P<.05) that FCM of cows injected with Ser-99 rbSt was greater than FCM for cows injected with either Gly-99 rbSt or native rbst. There was no suggestion of a difference in FCM of cows injected with Ser-99 rbSt compared to cows injected with Asp-99 rbSt or among cows injected with Gly-99 rbSt, Asp-99 rbSt, and native rbSt. FCM of cows administered 20 mg rbSt was statistically significantly greater than cows administered 5 mg.

Experimental Group	Mean FCM Relative to Days of Injection			Change[a] (kg/day)	Percentage Change for Days	
	1-3 pre	1-21 during	1-5 post		1-21 during[a]	1-5 post[b]
Control	27.6	26.1	26.1	-1.5	-5.4	0
Native 5 mg	28.2	28.0	26.8	-0.2	-0.7	-4.3
Native 20 mg	26.6	30.2	26.0	3.6	13.5	-13.5
Asp-99 5 mg	28.1	29.2	29.2	1.1	3.9	0
Asp-99 20 mg	24.5	29.0	27.0	4.5	18.4	-6.9
Gly-99 5 mg	29.3	29.4	17.2	0.1	0.3	-7.5
Gly-99 20 mg	26.4	29.8	28.6	3.4	12.9	-4.2
Ser-99 5 mg	28.8	31.0	30.4	2.2	7.6	-1.9
Ser-99 20 mg	28.9	33.5	31.6	4.6	15.9	-5.7

[a]Days 1 to 21 of injection relative to days 1 to 3 pre-injection.
[b]Days 1 to 5 post-injection relative to days 1 to 21 of injection.

Example 6

Activity of other position 99 analogs was demonstrated in a separate study from that reported in Example 5. Again, the parameter measured was to estimate differences in 3.5% fat corrected milk yield (FCM) of lactating dairy cows injected intramuscularly with native rbSt (clinical rbSt) that has asparagine at position 99, rbSt with proline substituted for asparagine at position 99 (Pro-99 rbSt), and

rbSt with glutamic acid substituted for asparagine at position 99 (Glu-99 rbSt). Holstein cows (35) were ranked from high to low milk yield based on milk yield on days 3 and 2 prior to initiation of rbSt injections. The cows were assigned randomly in replicates, based upon milk yield, to 7 experimental groups: no injection (Control), 5 mg and 15 mg Pro-99 rbSt daily, 5 mg and 15 mg Glu-99 rbSt daily, and 5 mg and 15 mg native rbSt daily. Cows were injected imtramuscularly in the semitendinosus muscle once daily for 7 days. Twice daily milk weights were recorded for three days prior to initiation of injection, during the 7 days of injections, and for five days after the last injection.

The dose averaged 5.04 mg, 4.88 mg, and 5.03 mg for cows assigned to receive the 5 mg dose of Pro-99, Glu-99, and native rbSt respectively. Cows assigned to receive 15 mg rbSt received 15.12 mg, 14.64 mg, and 15.10 mg rbSt for cows of the Pro-99, Glu-99, and native rbSt respectively. The percentage of early-early eluting rbSt, early eluting rbSt, oxidized rbSt, and post-oxidized rbSt appeared to be similar for native rbSt, Glu-99 rbSt, and Pro-99 rbSt. Statistical analyses of the FCM among Experimental Groups were based upon average FCM for days 1 to 7 of injections using the three days prior to initiation of injections as the covariate. The FCM for cows of the native rbSt, Pro-99 rbSt, and Glu-99 rbSt groups was 28.7, 28.8, and 28.8 (P=.95). Therefore there was no suggestion of a difference of FCM for cows receiving native rbSt, cows receiving rbSt with glutamic acid substituted for asparagine at position 99, and cows receiving rbSt with proline substituted for asparagine at position 99. FCM was significantly greater for cows of the 15 mg rbSt group than for cows of the 5 mg rbSt group. Upon cessation of rbSt injections, FCM of rbSt previously injected cows decreased at similar rates during the 5 days after cessation of rbSt injections.

Example 7

Samples of rbSt and rbSt position 99 analogs were examined for relative potency using the hypophysectomized rat growth bioassay. Two hundred hypophysectomized female Sprague Dawley rats weighing between 75 and 150 grams were used in each experiment. They were fed pelleted Purina Rat Chow ad libitum and watered with deionized and rechlorinated water. Room conditions were set at a temperature of 80° F and relative humidity of 50%. Air exchange was approximately

20 exchanges per hour and the photoperiod was 12 hours light and 12 hours dark, with the light cycle commencing about 6:00 a.m.

The rats were monitored for a 12-day preliminary period to allow for adaptation to environmental and feeding conditions. Body weights were obtained on four occasions between days one and twelve. Rats that showed weight losses of one gram per day or less or weight gains of 2 grams per day or less during the preliminary period were selected for injection with rbSt. The rats were ranked according to ascending magnitude of average daily gain (ADG), and seven blocks of 25 rats were created. Treatments within each block were randomly assigned.

rbSt treatments lasted 10 days. During this time, test compounds were injected twice daily for nine days, and body weights were monitored using a Mettler Model 3600 top loading balance equipped with lab-Pac programming which determines the weight of the animals while taking into account their movements.

Stock solutions of rbSt at 2 mg/ml were prepared in a buffer of 0.03 M sodium bicarbonate and 0.15 M NaCl at pH 9.5. To facilitate suspension of rbSt, the lyophilized preparations were first dissolved in this buffer at pH 10.8, then the pH was adjusted to 9.5 using 2 N HCl and brought to final volume using the stock buffer at pH 9.5 and filtered if necessary.

The stock solutions were diluted using stock buffer (pH 9.5) to solutions of 37.5, 75, 150, and 300 mg of protein/ml. The rats were injected subcutaneously twice daily with 100 μl of the respective solutions, and controls received 100 μl of buffer. The experiment lasted 10 days with average daily weight gain monitored.

A statistical analysis of the relative potency for the various test samples of rbSt was as follows: native rbSt with asparagine at position 99 served as an analytical standard and was assigned relative potency value of 1.00. This sample had a potency of 1.15 relative to a sample of pituitary-derived bovine somatotropin. The rbSt analogs in which aspartic acid, glutamic acid, glycine, proline, serine, and serine-serine were substituted for the asparagine at position 99, had relative potencies (respectively) of 2.30, 3.00, 2.96, 2.50, 3.06, and 3.03 relative to rbSt control. These values are significant at the 95% confidence level.

Example 8

The aqueous stabilities of three of the rbSt position 99 analogs, having glycine, serine, and aspartic acid substituted for asparagine, were compared with that of native (asparagine at 99) rbSt under two sets of incubation conditions. The first set of incubations was carried out in 50 mM sodium carbonate, pH 10.0 at ambient temperature (protein concentration 20 mg/ml) to simulate conditions similar to those encountered during the isolation and formulation of rbSt. The second set of incubations was carried out in a pH 7.4 Ringer's solution at 37°C (protein concentration 5 mg/ml) to simulate exposure to physiological conditions. All incubation samples were prepared aseptically. Samples were removed from the incubations over a three week period and stored at -20°C prior to analysis by isoelectric focusing, SDS-PAGE, and reversed phase HPLC.

The glycine 99 and serine 99 rbSt analogs had IEF patterns that were virtually indistinguishable from native rbSt prior to incubation while the IEF pattern of native asparagine 99 rbSt was shifted approximately 1.2 pH units lower due to the introduction of the negative charge at position 99. The major difference noted between the IEF patterns of incubated samples of native rbSt and the position 99 analogs was the rate at which more acidic rbSt species were formed. The IEF pattern of native rbSt degraded into more acidic rbSt species at a faster rate under both sets of incubation conditions than did the IEF patterns of the position 99 analogs. One additional difference between the IEF patterns of native rbSt and the analogs was the presence in incubated native rbSt samples of IEF bands that have been associated with a chain cleavage between residues 99 and 100 of the rbSt molecule. These bands were not observed in the IEF patterns of the position 99 analogs.

When analyzed under non-reducing conditions, the SDS-PAGE behavior of incubated samples of the position 99 analogs was identical to that of native rbSt. When examined under reducing conditions, however, the major rbSt fragment formed during the incubation of native rbSt was found to be absent in the position 99 analogs. Replacement of the asparagine residue at position 99 with either glycine, serine, or aspartic acid therefore eliminated the peptide bond cleavage between residues 99 and 100. The two peptides formed as a result of this cleavage in native rbSt are normally held

covalently intact by the disulfide bond between cysteine residues 53 and 164, thus the difference between the reduced and non-reduced samples.

Reversed phase HPLC was used to measure the amount of isoaspartate formation and chain cleavage which occurred at position 99 of the rbSt molecule since these species elute earlier upon reversed phase HPLC than native rbSt. rbSt which contained a chain cleavage between positions 99 and 100 of the rbSt molecule accounted for 8.5 area percent of native rbSt at the conclusion of the pH 10.0 incubation, and for 11.1 area percent of native rbSt at the conclusion of the pH 7.4 incubation. By comparison, the amount of the rbSt analogs which eluted in this position was less than 0.5 area percent. rbSt which eluted in the position of isoaspartic acid 99 rbSt accounted for 35.8 area percent of native rbSt at the conclusion of the pH 10.0 incubation, and for 49.9 area percent of native rbSt at the conclusion of the pH 7.4 incubation. Of the position 99 rbSt analogs, aspartic acid 99 rbSt showed the greatest formation of rbSt which eluted in this position: 3.8 area percent at the conclusion of the pH 10.0 incubation and 13.9 area percent at the conclusion of the pH 7.4 incubation.

Therefore, the position 99 analogs of the instant invention show superior aqueous stability relative to native asparagine 99 rbSt.

Example 9

Following the teachings of the preceding examples with appropriate modifications, similar analogs to porcine, human, ovine, horse, rat, monkey and avian somatotropins may also be produced by replacing the asparagine residues at position 99 (S.S. Abdel-Meguid, et al, Proc. Natl. Acad. Sci. USA, 84, pp. 6434-37 (1987). Because of the close sequence homology between mammalian somatotropins in this region of the molecule, the asparagine residue corresponding to bSt asparagine 99 is likely the third residue in a β-turn in each of these other somatotropins thus leading to isoaspartic acid formation and chain cleavage as with bSt. If this is found to be the case for other somatotropins by analysis of the products as set forth above, substituting an appropriate amino acid for the asparagine 99 would obviate the isoaspartate formation and chain cleavage.

Administration of the bSt analogs into dairy cattle is according to known methods using any route effective to deliver the required

dosage to the animal's circulatory system. Modes of administration include oral, intramuscular injections, subcutaneous injections and the use of timed-release implants. The preferred mode of administration is by subcutaneous injection using a timed-release implant. Appropriate vehicles for injections include physiologically compatible buffers such as sodium bicarbonate, sodium phosphate, or ammonium phosphate solutions. Timed-release implants are known in the art, e.g., U.S. patent 4,333,919.

The effective dosage range is from 1.0 to 200 milligrams per animal per day. The greater the amount of bSt given, the greater the resulting increase in growth, lactation or numbers of mammary parenchymal cells. Most preferably, the dosage range is from 5 to 50 milligrams per day.

Mammalian growth hormones are very similar in their amino acid sequences and hormones originating from one animal source can enhance the growth of other unrelated species of animals. For purposes of increasing growth rate of animals, the analogs of the present invention can be used to produce increased growth in the same animal species in which native bSt has been shown to have growth-related bioactivity such as bovines, sheep, rats, salmon and chickens. The preferred animals are bovine used for beef cattle such as bulls, heifers or steers.

Beef cattle are slaughtered just prior to reaching full maturity and size. The bSt analogs of the instant invention can be used to produce increased growth rates in beef cattle by administration any time between weaning until slaughter. The bSt are administered to beef cattle for a minimum of 30 days and for a maximum of 450 days depending upon desired time of slaughter. Animals used for veal are typically slaughtered at approximately 6 months of age and 10 to 30 mg/day of the bSt analog is administered up until the age of slaughter to effectuate desired increases in growth rate.

For purposes of increasing lactation in bovines, particularly dairy cows, the bSt analog is administered between 30 and 90 days postpartum and continued for up to 300 days. The bSt analog will also increase lactation in other commercial milk-producing animals such as goats and sheep.

CHART 1. Amino Acid Sequence Of Bovine Somatotropin

1
ala phe pro ala met ser leu ser gly leu phe ala asn ala val

20
leu arg ala gln his leu his gln leu ala ala asp thr phe lys

40
glu phe glu arg thr tyr ile pro glu gly gln arg tyr ser ile

60
gln asn thr gln val ala phe cys phe ser glu thr ile pro ala

pro thr gly lys asn glu ala gln gln lys ser asp leu glu leu

80
leu arg ile ser leu leu leu ile gln ser trp leu gly pro leu

100
gln phe leu ser arg val phe thr asn ser leu val phe gly thr

120
ser asp arg val tyr glu lys leu lys asp leu glu glu gly ile

leu ala leu met arg glu leu glu asp gly thr pro arg ala gly

140
gln ile leu lys gln thr tyr asp lys phe asp thr asn met arg

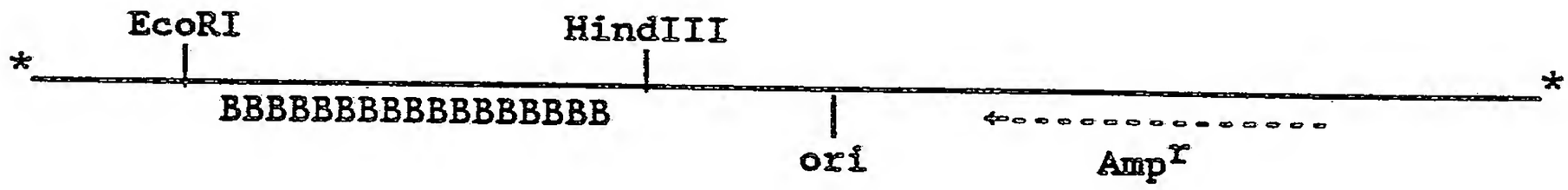
160
ser asp asp ala leu leu lys asn tyr gly leu leu ser cys phe

180
arg lys asp leu his lys thr glu thr tyr leu arg val met lys

190
cys arg arg phe gly glu ala ser cys ala phe

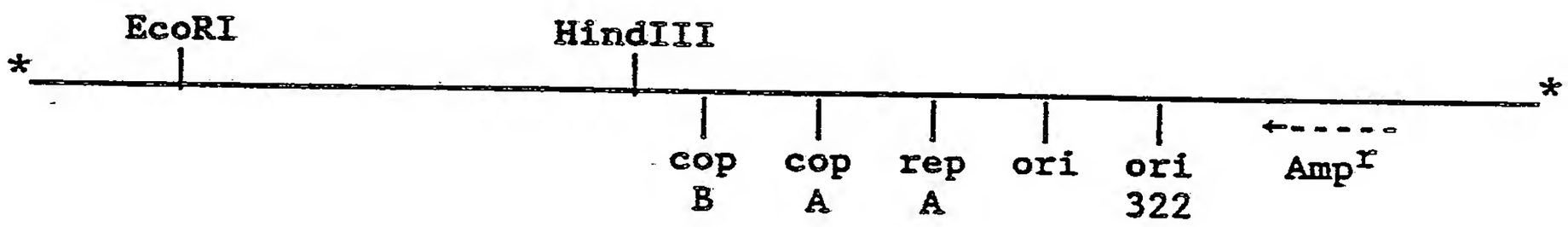
CHART 2. Cloning of the m4-99ser gene into the pURA Vector

1) A plasmid containing the m4-99ser gene is digested with EcoRI and HindIII to produce Fragment 1.

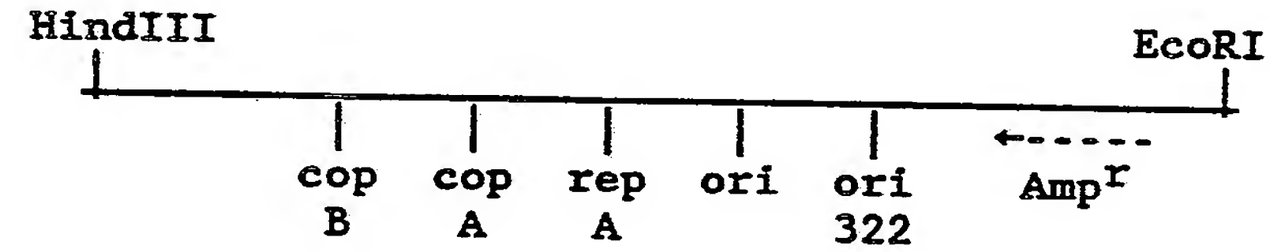


Fragment 1

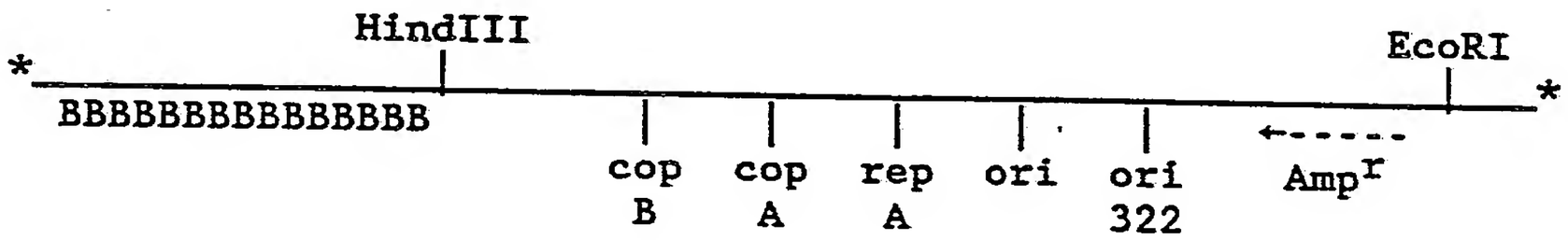
2) Plasmid pURA-m4 is digested with EcoRI and HindIII to produce Fragment 2.



Fragment 2



3) Fragments 1 and 2 are then ligated together to produce plasmid pURA-99ser.



B = bSt m4-99ser gene

CHART 3. Oligonucleotides Used for Constructing rbSt Analogs

C-ST 84 (gly)	5'-GTCTTCACCGGTAGCTTGGTG
C-ST 88 (ser)	5'-GTCTTCACCTCTAGCTTGGTG
C-ST 89 (pro)	5'-GTCTTCACCCCGAGCTTGGTG
C-ST 90 (asp)	5'-GAGTCTTCACTGATAGCTTGGTG
C-ST 91 (glu)	5'-GAGTCTTCACTGAAAGCTTGGTG
JM 23 (ser-ser)	5'- AGCAGAGTCTTCACCTCTTCCTCTTTGGTGTTTGGCACC
JM 57 (ser-asp)	5'-TCAGCAGAGTCTTCACCTCTGACTCCTTGGTGTTTGGCACCTCGG

CLAIMS

1. An animal somatotropin in which the asparagine residue corresponding to residue 99 of the native somatotropin as exemplified in Chart 1, is replaced with at least one different amino acid selected from the group consisting of:

(a) proline;

(b) serine;

(c) glycine;

(d) serine-serine; or

(e) serine-aspartic acid.

2. A mammalian somatotropin according to claim 1 wherein the asparagine is replaced with proline.

3. A mammalian somatotropin according to claim 1 wherein the asparagine is replaced with serine.

4. A mammalian somatotropin according to claim 1 wherein the asparagine is replaced with glycine.

5. A mammalian somatotropin according to claim 1 wherein the asparagine is replaced with serine-serine.

6. A mammalian somatotropin according to claim 1 wherein the asparagine is replaced with serine-aspartic acid.

7. An animal somatotropin according to claim 1, selected from the group consisting of bovine, porcine, fish, ovine, horse, rat, monkey, and human somatotropins.

8. An animal somatotropin according to claim 1, which is bovine somatotropin.

9. An animal somatotropin according to claim 1, which is bovine somatotropin.

10. An animal somatotropin according to claim 3, which is bovine somatotropin.

11. An animal somatotropin according to claim 4, which is bovine somatotropin.

12. An animal somatotropin according to claim 5, which is bovine somatotropin.

13. An animal somatotropin according to claim 6, which is bovine somatotropin.

14. An animal somatotropin according to claim 7, which is bovine somatotropin.

15. A method for enhancing the growth of an animal which comprises administering to the animal an effective amount of a somatotropin of claim 1.

16. The method of claim 15 wherein the animal is a bovine.

17. A method for increasing milk production in a cow comprising administering to the cow an effective amount of an animal somatotropin of claim 1.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/05447

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 13/00, C 12 N 15/18, A 61 K 37/36

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	WO, A2, 87/01708 (THE UPJOHN COMPANY) 26 March 1987, see the whole document --	1,6,7, 13,14-17
A	WO, A1, 88/07091 (AMGEN INC.) 22 September 1988, see the whole document --	1-17
A	EP, A1, 0085036 (MONSANTO COMPANY) 3 August 1983, see the whole document --	1-17
A	EP, A2, 0089666 (GENENTECH, INC.) 28 September 1983, see the whole document --	1-17

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th April 1990	27. 04. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. Peis M. PEIS

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/05447

SA 33755

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/02/90 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A2- 87/01708	26/03/87	NONE		
WO-A1- 88/07091	22/09/88	NONE		
EP-A1- 0085036	03/08/83	AU-B-	556347	30/10/86
		AU-D-	1050883	28/07/83
		CA-A-	1188221	04/06/85
		JP-A-	58124720	25/07/83
EP-A2- 0089666	28/09/83	GB-A-B-	2116980	05/10/83
		JP-A-	59001428	06/01/84
		US-A-	4446235	01/05/84
		US-A-	4665160	12/05/87
		US-A-	4670393	02/06/87